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Research Article

Evaluation on Mercury, Cadmium, and Lead in the Hair Sample as an Indicator of Autism for Children

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ABSTRACT
Adequate knowledge is required to determine the toxicity degree of heavy metals such as cadmium, lead and mercury in the human body causing adverse effects and chronic diseases like autism for kids. Microwave-assisted digestion, hair sampling using HNO_3 solution combined with H_2O_2 hydrogen peroxide and the measurement by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) were used for the element determination. A study conducted on hair samples of 40 individuals ranging from 5 years - to 17 years (school age) living in Malang in 2017 aimed to determine the level of heavy metals (Cd, Pb and Hg) across autism and normal children technique (ICP-MS). The results of replicate analysis shows the following mean concentrations (µg/g), Autsim cd 4.34 ± 0.06, Pb 44.75 ± 0.56 Hg 1.70 ± 0.078 and Normal Cd 2.63 ± 0.04, Pb 48.49 ± 0.80 Hg, 1.54 ± 0.13, indicating a higher concentration compared to the standard certified value GBW07601. The higher concentration of all the heavy metals (Cd, Pb, and Hg) occurred in both normal children and children with autism disease. The comparison noted that cadmium percentage was higher than elemental mercury in children with autism and vice versa in normal children. Yet, the concentration of both elements was higher in children with autism. Nonetheless, lead was found higher in normal children. It is also supported by several studies confirming the relationship between organic mercury and nervous system including autism disease. On the other hand, in this study, we found no statistically significant differences in the concentrations of heavy metals (Pb, Cd, and Hg,) between the normal children and children with autism disease.

Keyword: heavy metal, autism, hair, children, indicator.

INTRODUCTION

Heavy metals are the main threat to human health, these metals have been extensively studied and their effects on human health regularly have been reviewed by international bodies such as the World Health Organization (WHO). Although several adverse health effects of heavy metals have been known over a long period of time, the exposure to heavy metals continues to happen. Toxic elements can be very harmful even at low concentration when ingested for a long time[1]. Toxic heavy metals that should be concerned at most are cadmium, lead, and mercury. The exposure to these metals is a continuous daily process as they can be found at the place of work, in potable water, in food, and in the air. The toxicity of metals stems from the fact that they are biologically non-degradable and have the tendency to accumulate in water, sediment and fish[2]. Metal contamination of the environment results both from natural sources and industrial activities. Metals in soil and water may enter the food cycle with an additional contribution from air[3]. The general population is primarily exposed to mercury through food, fish is a major source of methyl mercury exposure and dental amalgam. In addition, mercury also derives from Hg-containing pharmaceuticals administered to the mother while the child is growing in utero and injected organic-Hg from Thimerosal. Thimerosal is an organic-Hg compound (49.55% Hg weight) added to vaccines as a preservative, typically at concentrations from 0.005% to 0.01% (12.5 µg Hg or 25 µg Hg per 0.5 mL vaccine dose)[4]. Major sources of Pb pollution are exhaust gases of petrol engines, which account for nearly 80% of the total Pb in the air. Soils located near Pb mines may contain high Pb content of 0.5%. Apart from minerals, sources of Pb are pesticides, fertilizer impurities, emissions from mining and smelting operations, as well as atmospheric fallouts from the combustion of fossil fuels. Either organic or inorganic form of lead is absorbed through the lungs and gastrointestinal. Organic lead can also be absorbed through a skin. Inhalation exposure is more common in an occupational setting. Up to 94% of the body burden contained lead is in bone, where it has a half-life of years to decades. The lead would inhibit calcium-binding protein[5].

Children and adolescents are more vulnerable to heavy metal poisoning, and can suffer from improper mental

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development and low intelligent quotients (Hope 2017). One of the disorders that result from the interplay between genetic susceptibility and exposure to environmental hazards is Autism Spectrum Disorder (ASD)[6]. Autism is a serious neurodevelopment al disorder characterized by impairments in social interaction, verbal and nonverbal communication, and other restricted behaviors. The number of children reported with autistic spectrum disorders (ASDs) has increased dramatically during the last 10 years[7]. Toxic heavy metals as mercury, aluminum, and lead represent potential environmental hazardous factors that might lead to the ASD development[8]. Moreover, most of the studies confirmed that here is a strong relationship between chronic diseases (such as autism) and the accumulation of organic mercury compound during injection for children[4]. Mohamed et al.,[9] analyzed the relation between intoxication with heavy metals and ASD. They assessed the level of aluminum, lead, and mercury in hair samples of 100 autistic children compared to age and sex matched those of healthy controls. Hair sampling was chosen because it is seen as the best non-invasive maneuver indicating the level of a given mineral in the body[10]. They discovered significantly higher levels of all measured heavy metals in autistic children compared to controls with positive relation to exposure to risk factors for heavy metals intoxication like extensive antenatal consumption of fish, maternal smoking, cooking in aluminum pots, and anti-D intake. Autistic children are defective in metabolizing sulfur compounds that cause significant reduction of their abilities to detoxify heavy metals and increases their toxicity, with subsequent negative impact on the development of the brain and normal CNS functions[11].

Mercury is neurotoxicity and it has been shown to specifically target long-range axons in the brain. In addition, Hg is able to potentiate the effects of other xenobiotics such that a combined exposure from different sources should be seriously concerned because cumulative effects from various sources could potentially increase a child's Hg body burden[4]. Moreover, lead also causes significant oxidative stress and lipid peroxidation either directly or indirectly[12], alters synaptic pruning[13], interferes with glutamate; an excitatory neurotransmitter that is thought to be related to normal development of neurons[14], lowers hippocampal expression of protein kinase C (PKC)[15], and produces volume loss in vital portions of the prefrontal cortex[16]. Accordingly, many authors postulated that lead is never safe whatever its level is and can result in aberrant learning and defective neurobehavioral at levels as low as 10 μg/dl[17].

In addition, humans are susceptible to toxicity sources, primarily Cadmium. Gastrointestinal ingestion of Cd through food or drinking water is the major route of intake for this metal. In addition, this metal can also derive from mining and metallurgical activities of tanning industries. However, still, Cigarette smoke is the largest source of Cd exposure because each cigarette can contain up to 6.67 μg Cd, and 40-60% of it generally passes through the pulmonary epithelium into systemic circulation. Chronic Cadmium intoxication results mainly in renal disease but acute Cadmium exposure primarily results in liver accumulation and hepatocellular damage, occurring after the acute exposure to inorganic forms of Cadmium[18].

Another emerging issue related to the environmental heavy metal toxicity that needs to be seriously taken into account is the increasing incidence (1 out of 150 children) of autism spectrum disorder recently reported by Center for Disease Control. High blood levels of heavy metals in autistic children suggest a possible relationship to heavy metal exposures such as Pb, Cd, and Hg. The potential role of Hg in the disease process gains some support from the possible relationship with a single agent in the environment with the incidence of a neurodegenerative disease process including autism[19]. Despite the great number of studies being made concerning cellular and the molecular dysfunctions associated with autism[20].

There are several biological specimens that can be obtained in a non-invasive way – saliva, hair, and nails. That kind of sample collection does not require special facilities and close supervision of private functions. One of the crucial points in non-conventional matrices is the possibility to extend the time window of detection over weeks or months, as the assessment of chronic disease and cumulative exposure monitoring performed through hair samples. These specimens can contain proteins, lipids, various enzymes, carbohydrates, which interfere with the analyte determination. The type of sample is one of a few factors that should be taken into account in choosing an analytical technique prior to final analysis, isolation and/or pre-concentration[21]. Hair may constitute a suitable material for the evaluation of environmental metal exposure, including mercury, lead, and cadmium. The type of environment is connected with the place. The effect of the environment on concentrations of mercury, lead, and cadmium in human hair has been shown in several studies by many references. The content of analyzed metals in hair is significantly affected by the diet or some drugs. The place of residence also has an effect on the concentrations of these metals in hair. Inhabitants of rural areas have lower metal contents in their hair than those living in big cities. The height and weight of examined individuals do not have an effect on concentrations of lead and cadmium in their hair[22]. Hair analysis is inexpensive and fast; it also detects and measures the content of heavy metals and minerals of the hair. The Global Environmental Monitoring System (GEMS) of the United Nations Environment Program selected human hair as one of the important monitoring materials for worldwide biological monitoring of pollution[23]. Hair is also a stable biological material, which can easily be stored at room temperature for a long time without a change in its composition[24]. Furthermore, the concentrations of metals in hair sample are high compared with those in body tissues or fluids. This characteristic makes hair an attractive bio monitoring substrate[25].

These concentrations may be an indicator of some chronic diseases, such as neurodegenerative diseases or other chronic diseases. Measuring levels of metals in human bodies is usually done by analyzing human fluids such as blood and urine, but the results reflect a transient situation.

Therefore, the metal content in hair can reflect the body status over a long period of time, including exposure to metals for certain period of time. Hair analysis is a promising tool for routine clinical screening and making a diagnosis of heavy metal exposure and essential trace element states in the human body. For the analysis of hair, the solid samples are transferred by solubilization through digestion into a liquid phase. Small molecular solvents and molecules with hydrophobic groups appear to have a higher affinity for hair. The digestion of the sample is often the most important step in the chemical analysis, which can influence the further results. The majority of procedures involve the use of a single or some combinations of oxidizing acids, high (hot) temperature or room temperature, diluted or concentrated acids (HCl, H_2SO_4, HNO_3, $HClO_4$, HF, H_2O_2 etc). Nowadays, microwave-assisted wet digestion procedures in closed vessels are widely used in routine by several laboratories and can be considered as the state-of-the-art for sample digestion, especially for samples containing high amounts of organic compounds. However, when concentrated reagents are used, a high concentration of acid remains in digests and a subsequent dilution step for reducing the excessive acidity may be necessary to avoid interferences in some determination techniques[26]. There are many analytical atomic spectrometry methods available for element determination, such as flame atomic absorption spectrometry (FAAS), glow discharge atomic absorption spectrometry (GD-AAS), graphite furnace atomic absorption spectrometry (GF-AAS), flame atomic emission spectrometry (FAES), glow discharge atomic emission spectrometry (GD-AES), inductively coupled plasma atomic emission spectrometry (ICP-AES), glow discharge atomic fluorescence spectrometry (GD-AFS), inductively coupled plasma atomic fluorescence spectrometry (ICP-AFS), glow discharge mass spectrometry (GD-MS), and inductively coupled plasma mass spectrometry (ICP-MS) have been used for many years for the determination of elements since they met needs required in analytical applications[27]. Performing microwave-assisted digestion and using HNO_3 solution combined with H_2O_2 hydrogen peroxide are seen to be effective strategy that can be implemented without decreasing the digestion efficiency. In common, a mixture of HNO_3 and H_2O_2 is used for botanic, biological and food samples. MW-assisted digestion in under pressure-closed vessels is the most commonly applied. It offers safety radiation, versatility, energy control and a possibility for an addition of solutions during digestion. Measurements of elements in various materials are the only way to get the knowledge about their composition. In fact, the key to the success of the whole analysis is the selection of the sample preparation method. An aappropriate sample preparation method allows obtaining required and reliable information about element concentration of samples.

In this study, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used for the analysis of three heavy metals (Pb, Cd, and Hg) in human hair due to its high sensitivity, low detection limit and minimal analyte loss of interest during digestion processes in the conventional method of atomic absorption spectroscopy (ASS) have been used for many years for the determination of elements since they met needs required in analytical applications[28].

EXPERIMENT

In 2017, this study was conducted on hair samples of 40 individuals living in Malang. These people represented a wide range of age from 5 years - to 17 years. They were selected for the study in two schools. Analyses were conducted on the sample hair not subjected previously to hairdressing procedures. Hair specimens were collected from both schools covering first 20 samples from "Daarul Fikri" Malang, second 20 samples from *"*SLB AUTIS LABORATORIUM UM*"* Malang. An interview was conducted using a chart prepared in advance, including personal data for children.

Chemicals and instrumentation

Glasswares, a microwave oven (MODENA,Indonesia) used for experiments, some storage bottles of 5,10 and 50 ml, wash bottles, 2 ml serological pipettes and pipette pump(Merck, Germany). determined by inductively coupled plasma mass spectrometry ICP-MS(NexIon^{TM},China) were used for measurements and digestion performed under inert atmosphere. Experiments were carried out using whole four specimens' hair of 3-5 ml for each step. Samples were weighed using an analytical balance, distilled water, and tools commonly used in laboratories. Analytical grade nitric acid (65 %, density 1.512 g/mL, hydrogen peroxide H_2O_2 of 30%) from Merck (Germany).

Procedure reaction

Analyses were conducted on hair not subjected previously to hairdressing procedures. Hair specimens collected from the schools weighted 0.5 g and the length was cut to be 0.3 -0.5 cm. After cutting, the hair samples were washed two times with acetone and four times with deionized water. After that, those samples were put in the oven around 24 hours at 70^o C until they were dry and clean. In the next step, 0.2 g hair samples were taken in 50 ml polypropylene centrifuge tubes and then added with 4 ml concentrated nitric acid of 65% HNO_3 and 2 ml concentrated hydrogen peroxide H_2O_2 of 30% mixed at a ratio of 2:1. Then, the hair samples were put in the microwave with the temperature of 110 ◦C for 15 min and took 30 min of waiting until the samples were cool. The samples were then put in the refrigerator. After the samples digestion of 5ml in 50 ml were done, the samples in polypropylene centrifuge tubes were moved to 15 ml tubes and added with 10 ml nitric acid 1M until it reached the volume of 15 ml. The content of lead, cadmium, and mercury in the solution was determined by Inductively Coupled Plasma Mass Spectrometer (ICP-MS) equipped. The precision and repeatability of the method were verified (using many uniform hair samples) and the recovery of cadmium and lead was analyzed by adding a certain amount of the standard solution to many uniform hair samples at the same time. The results were developed statistically using Student's t-test and values of correlation coefficients.

Heavy Metal Analysis

We have assessed Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as a method for the quantitative detection. We also have established a linear relationship between the unconjugated concentration and the mass of cadmium, lead, and mercury detected by ICP-MS. The method employed for analyzing the content of the heavy metals was using the (Inductively Coupled Plasma Mass Spectrometry) ICP- MS according to the Lambert beer's law. This law explains that the amount of light absorbed is proportional to the amount of heavy metal content in the material.

The real concentration of heavy metals can be discovered using this formula:

$$\text{Real concentration} = \frac{(D - E) \times F_P \times V}{W(g)}$$

Description:

D: sample concentration μg L^{-1} from the ICP-MS reading;

E: blank sample concentration μg L^{-1} from the ICP.MS reading;

Fp: dilution factor;

V: final volume of the prepared sample solution (mL);

W: sample weight (g).

Statistical Data Analysis

The analysis of *t-test* variance was performed on each brand of hair to find out if there was a significant variation in the concentrations of heavy metals in different colors of each brand. To compare the heavy metal concentrations in hair samples of normal and autistic children, statistical software and principal component analysis were applied. The results were determined as Mean (μg)±SE or Mean(μg/ml) ± SE from two replicates in each test.

Data were presented in tables (Table 1). The test was used to analyze the relationship between the chronic diseases and heavy metals. All values < 0.05 would be considered not significant. Graphs were made to illustrate the heavy metal effects in the hair samples. Tables were constructed to illustrate the relationship between heavy metals and the chronic diseases.

RESULT AND DISCUSSION

Hair analysis result

The current study, a biomonitoring of three hair heavy elements (Cd, Hg and Pb) in school children from Malang city is reported , has been assessed controls In total, 40 hair samples were analyzed using double focusing sector field inductively coupled plasma mass spectrometry after microwave- assisted sample digestion with nitric acid. For the determination of cd , hg and Pb inductively coupled plasma mass spectrometry was employed. For the validation, a reference material and spiked hair samples were analyzed. The data obtained was processed using parametric statistics and factor analysis. Determined concentrations of heavy elements were in agreement with the previously published results on chemically polluted areas .In the case group, linear correlations between Cd, Hg, and Pb were observed. Additionally, the gender, distribution of the sample are shown in tables 2. Female participants accounted .Female participants accounted for

Table 1: Condition of analysis of number of sample hair

Valid	Frequency	Percent	Valid Percent	Cumulative Percent
Normal	20	50.0	50.0	50.0
Autism	20	50.0	50.0	100.0
Total	40	100.0	100.0	

Table 2: Distribution and mean analysis of heavy metal in male and female samples.

Valid	Frequency	Percent	Valid Percent	Cumulative Percent
Male	27	67.5	67.5	67.5
Female	13	32.5	32.5	100.0
Total	40	100.0	100.0	

32.5% of the collected hair samples while 67.5% of the hair samples were collected from males, several gender differences in Lead, Cadmium and Mercury content were observed within each group.

The primary aim of this study was to assess the relationship between concentrated heavy metals in the hair as an indicator of autism disease in children (teens) aged 3–17 years. The heavy metals being studied were lead (Pb), cadmium (Cd), mercury (Hg).

Heavy Metal (Cd, Pb, and Hg) Concentration in Hair Sample

Based on Table 4. it is pointed out that the heavy metals had much higher concentrations compared to the standard certified values of GBW07601. The higher concentrations of all the heavy metals (Cd, Pb, and Hg) in the samples occurred for both normal children and autistic children due to the pollution in the environment of Malang. In any case, this indicator was an important need for the follow-up and monitoring of heavy metals such as cadmium, lead, and mercury in biological samples. Furthermore, the high concentrations of the elements in the body could lead to serious and chronic diseases, such as autism in children.

Relationship between Heavy Metals and Autism Disease

Scientist have found that heavy metals such as mercury, cadmium and lead affects chemical synaptic transmission in the brain and the peripheral and central nervous system (CNS) (Laila Y 2005).Those elements can lead to

Table 3: Influence of gender on elemental concentrations (mg/g) in human hair

Category	Pb	Pb error	Cd	Cd error	Hg	Hg error
Male Children with Autism	43.023	±0.52	4.19	±0.055	1.31	±0.08
Male normal Children	55.4	±1.07	3.82	±0.06	1.38	±0.049
Female Children with Autism	42.82	±0.58	1.64	±0.04	1.76	±0.2
Female normal Children	49.91	±0.7	4.8	±0.1	2.87	±0.07

Table 4: Concentrations of Elements (µg/g) in Standard Reference Materials by ICP-MS After Microwave Digestion

Element	Human hair GBW07601 (Certified Value)	Observed	
		Autism	Normal
Cd	0.11±0.02	4.34±0.06	2.63±0.04
Pb	8.8±0.9	44.75±0.56	48.48±0.80
Hg	0.36±0.05	1.70±0.078	1.54±0.13

Table 5: Ratio of Lead, Cadmium, and Mercury Concentrations in the Normal State of Normal and autistic children determined using the Linest function in Excel

Elements	Pb	Cd	Hg
Autism	48%	62%	56%
Normal	52%	38%	44%

disruption of brain and cellular calcium levels that significantly affect several functions, such as calcium dependent neurotransmitter release, which results in depressed level of serotonin, norepinephrine and acetylcholine.Those neurotransmitters are related to mood and motivation. The comparison between the ratios of heavy elements in the hair samples of normal and autistic children noted that cadmium was present in children with autism of 62% and in normal children of 38%. Meanwhile, elemental mercury was present in children with autism of 56% and in normal children of 44%. This indicated that both elements presented in autistic children were higher than in normal children. These percentage might indicate a relationship between cadmium, mercury and autism disease. It is also supported by several studies confirming the relationship between organic mercury and nervous system including autism disease. In contrast, the lead element presented in normal children was higher than in autistic children, which amounted to 52% in normal children and 48% in children with autism. This result confirmed the lack of correlation between the lead element in hair and the disease of the nervous system. According to the tables 5, the concentrations of heavy metals (Pb, Cd, and Hg) in the hair samples of both normal and autistic children did not show a significant result. Besides, there were no other statistically significant differences in the concentrations of heavy metals in both groups.

CONCLUSION

Using ICP-MS, in this study, there is no statistically significant differences found in the concentrations of heavy metals (Pb, Cd, and Hg,) between the normal and autistic children. The result shows the presence of all the metals in relatively large amounts with as having the highest concentration between the two genders. The difference between male and female concentration could be due to individual differences in exposure to heavy metal load as a result of habitual or environmental factors. Also results from the current study, demonstrated significantly

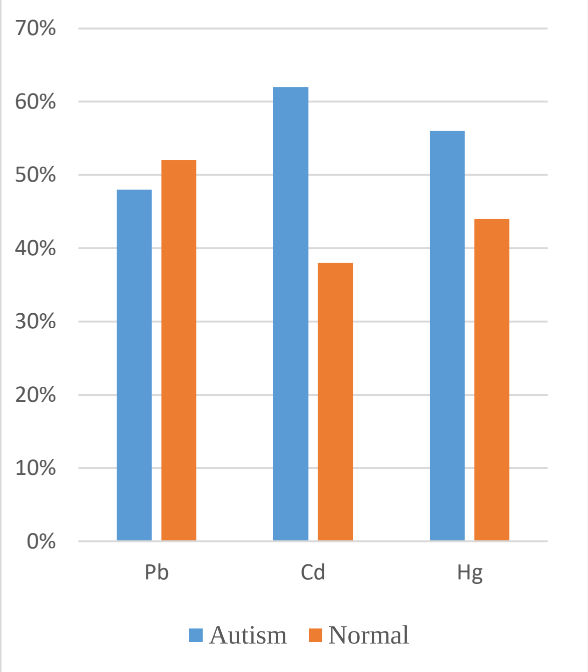

Figure 1 Ratios of Lead, Cadmium, and Mercury Concentrations in the Normal State of Normal and Autistic Children

higher levels of toxic heavy metals in hair samples from children with autistic spectrum disorders, such as, mercury, cadmium. The most likely explanation is due to exposure to toxic metals through drinking water, food or drugs like vaccine. The possible explanation for this is that autistic children might lack the ability to detoxify toxins, resulting in an accumulation of toxic substances in the body, and leading to alterations in biochemical processes taking place in the body. Therefore, heavy metal chelating therapy is necessary in some cases.

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